Knocking out a specific tRNA species within unfractionated *Escherichia coli* tRNA by using antisense (complementary) oligodeoxyribonucleotides

Takayuki Kanda[a], Kazuyuki Takai[a,*], Shigeyuki Yokoyama[b], Hiroshi Takaku[a]

[a]*Department of Industrial Chemistry, Faculty of Engineering, Chiba Institute of Technology, 2-17-1, Tsudanuma, Narashino, Chiba 275-0016, Japan*
[b]*Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

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Abstract Methods for the preparation of an *Escherichia coli* tRNA mixture lacking one or a few specific tRNA species can be the basis for future applications of cell-free protein synthesis. We demonstrate here that virtually a single tRNA species in a crude *E. coli* tRNA mixture can be knocked out by an antisense (complementary) oligodeoxyribonucleotide. One out of five oligomers complementary to $tRNA^{Asp}$ blocked the aspartylation almost completely, while minimally affecting the aminoacylation with other 13 amino acids tested. This 'knockout' tRNA behaved similarly to the untreated tRNA in a cell-free translation of an mRNA lacking Asp codons.

Key words: Cell-free protein synthesis; Antisense oligodeoxyribonucleotide; tRNA; Aminoacylation; *Escherichia coli*

1. Introduction

Methods of cell-free protein synthesis driven by crude extracts (S30) from *Escherichia coli* have been applied to the preparation of proteins containing unnatural amino acids at specific positions [1,2]. This technique has enabled detailed mutagenic analyses of protein structure-function relationships [2]. In this method, aminoacyl-tRNA molecules charged by chemical methods [1,3] are used to decode a stop codon placed at an intended position on an mRNA. Specific stable-isotope labeling of proteins for efficient NMR signal assignments was also achieved by using an enzymatically aminoacylated tRNA that read the amber stop codon [4].

Stop codons are convenient because the readthrough product indisputably has the unnatural/labeled amino acid at the intended position. If a sense codon, instead of a stop codon, is used for unnatural/labeled amino acids, the byproduct, resulting from the reading of the codon by the competing, intrinsic tRNA, has nearly the same size as the intended protein. This problem was overcome by the use of four-base codons that induce +1 frameshifts when recognized by the complementary four-base anticodons [5]. By this strategy, several unnatural amino acids were introduced into proteins [6,7].

However, it is difficult to introduce unnatural/labeled amino acid residues into multiple sites of a single polypeptide chain by these methods, because the aminoacyl-tRNA competes, at each site, with another component of the S30-based translation reaction: in the case of the stop codon system, the peptide chain release factor terminates the synthesis. In the case of the four-base codon system, the intrinsic natural aminoacyl-tRNA(s) insert(s) the natural amino acid. It may be difficult to avoid these limitations when using crude extract-dependent systems.

On the other hand, reconstituted cell-free translation systems have also been developed for different purposes [8]. In these methods, protein synthesis is completely dependent on the addition of tRNAs. Thus, if an unnatural/labeled aminoacyl-tRNA that reads a sense codon and an *E. coli* tRNA mixture lacking the ability to read the sense codon, but not the other codons (a 'knockout' tRNA mixture), are used in this translation system, all of the synthesized protein molecules will have the unnatural/labeled amino acid at the position(s) of the sense codon. Therefore, a technique for the preparation of the 'knockout' tRNA mixture may help to overcome the limitation in the number of unnatural/labeled residues that can be incorporated into proteins.

The 'knockout' tRNA mixture can be prepared, in principle, if one or a few tRNA species within the crude *E. coli* tRNA mixture can be inactivated specifically. As for the specific inactivation of RNA molecules, antisense strategies have been tested intensively. Although the major targets of the antisense strategies are mRNAs, tRNA molecules have also been tested as targets [9,10]. In particular, an RNA molecule complementary to the 5′-half of the initiator methionine tRNA binds specifically to the target tRNA in a wheat germ translation system [9]. In addition, all of the *E. coli* tRNA species have been specifically detected by Northern blot hybridization methods [11], and essentially any tRNA species within a crude tRNA mixture can be isolated based on oligonucleotide hybridization [12].

In the present study, we tested whether the activity of a single tRNA species in unfractionated *E. coli* tRNA can be removed selectively through annealing with the antisense (complementary) DNA molecules. The target we chose was $tRNA^{Asp}$ [13] (Fig. 1), for this tRNA is a unique Asp acceptor species [14], and thus can be assayed by aminoacylation. We also tested the effects of RNase H, which may irreversibly inactivate the target tRNA hybridized with the antisense DNA.

2. Materials and methods

2.1. Oligonucleotides

The sequences of the five unmodified antisense oligodeoxyribonucleotides are listed in Table 1. These were synthesized by the standard procedure with the use of an Applied Biosystems Model 392 DNA/RNA Synthesizer, and were purified by denaturing 20% polyacryl-

*Corresponding author. Fax: (81) (474) 78-0422.
E-mail: takai@ic.it-chiba.ac.jp

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amide gel electrophoresis followed by electroelution and ethanol precipitation.

2.2. Annealing and RNase H digestion

An unfractionated *E. coli* tRNA mixture was prepared as described [15]. This (20 μg) was mixed with an oligonucleotide in a solution (42 μl) containing 2 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, and 5 mM NaCl, and was incubated at 80°C for 5 min with or without 7 units of RNase H from *Thermus thermophilus* HB8 (Toyobo, Japan). The samples were gradually cooled to room temperature. In some experiments described below, the buffer and/or the salt conditions were changed.

2.3. Measurement of amino acid acceptor activity

The tRNA was mixed with an S100 fraction from *E. coli* [16] in a buffer (50 μl) containing 50 mM HEPES-KOH, pH 7.2, 16 mM $MgCl_2$, 2 mM ATP, potassium salt, 10 mM KCl, and 740 Bq [^{14}C]amino acid (specific activities were 185–222 kBq per carbon atom), and the mixture was incubated at 37°C for 10 min. The radioactivity in the cold 5% trichloroacetic acid precipitate was counted on a Whatman 3MM filter disk with an LSC-5100 liquid scintillation counter (Aloka, Japan).

2.4. Cell-free translation

We changed the unique TCT codon in the coding sequence of the plasmid pART23GGC3TCT [17] into AGA, originally for another purpose (submitted for publication). This plasmid was digested with the restriction enzyme *Bgl*I, and was transcribed with T7 RNA polymerase as described [17]. The transcript was purified by polyacrylamide gel electrophoresis followed by electroelution and ethanol precipitation. The cell-free translation (50 μl) contained 55 mM HEPES-KOH, pH 7.5, 1.7 mM DTT, 275 μM GTP, 26 mM phosphoenolpyruvate, potassium salt, 1.2 mM ATP, 1.9% polyethyleneglycol 8000, 34 μg/ml folinic acid, calcium salt, 6.9 mM ammonium acetate, 1 mM spermidine, 7.5 mM magnesium acetate, 210 mM potassium glutamate, 0.37 mM each of valine, threonine, alanine, glycine, tyrosine, methionine, glutamine, and lysine, 44 kBq/ml [^{14}C]arginine, one-sixth volume of S30 extract prepared as described [17], 40 μg/ml of the mRNA, and the treated or untreated tRNA. Radioactivities in the alkali-resistant, acid-insoluble fraction were measured with a liquid scintillation counter as described [17].

3. Results and discussion

3.1. Specific inactivation of tRNA^{Asp}

We first measured the Phe and Asp acceptor activities of the unfractionated *E. coli* tRNA before and after annealing in the presence of the oligonucleotides targeted to tRNA^{Asp} (Fig. 2). The Asp-3 oligonucleotide effectively impaired the Asp acceptor activity at a concentration of 1 μM, without affecting the Phe acceptor activity. Comparable experiments, with an annealing buffer of 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 50 mM NaCl, yielded essentially the same results (data not shown). Asp-4 had activity comparable to Asp-3. Asp-5 was also effective, but required a higher concentration for the inactivation of tRNA^{Asp}, and was not very specific, since the Phe acceptor activity was also lost at high Asp-5 concentrations. Asp-1 and Asp-2 did not impair tRNA^{Asp} activity, even at concentrations of 64 μM.

Table 1
Sequences of antisense oligodeoxyribonucleotides[a]

Name	Sequence	Target[b]
Asp-1	5′-TAACCGACTGAACTACCGCTCC-3′	1–21
Asp-2	5′-GTGACAGGCAGGTATTCTAACCGACT-3′	14–38
Asp-3	5′-GACCCCCTGCGTGACAGGCAGGT-3′	26–48
Asp-4	5′-ACCCGCGACCCCCTGCGTGACAG-3′	32–54
Asp-5	5′-TGGCGGAACGGACGGGACTCGAA-3′	54–76

[a]All oligonucleotides are unmodified, phosphodiester DNAs.
[b]tRNA residue numbers that correspond to the 3′- and 5′-ends of the oligonucleotides are shown (Fig. 1).

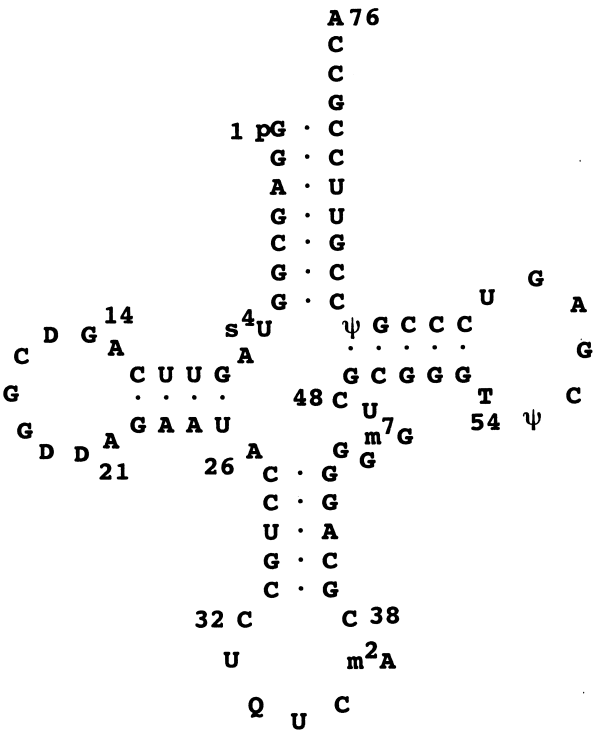

Fig. 1. The nucleotide sequence of tRNA^{Asp} [13] from *E. coli* and the target sites of the complementary oligonucleotides used in this study. The numbers show the positions corresponding to the 5′- or 3′-terminus of the antisense oligonucleotides used in this study (Table 1).

In order to estimate the specificity of the targeted inactivation, we tested the acceptance of 12 other amino acids for the Asp-3-treated tRNA mixture (Fig. 3). The results showed that only the Asp acceptor activity was impaired. Although this does not strictly mean that only tRNA^{Asp} is inactivated, it is expected that almost all codons, except for Asp codons, can be translated with this Asp-3-treated tRNA mixture.

3.2. RNase H improves the efficiency of the tRNA^{Asp} inactivation

The binding of oligonucleotides to tRNA molecules may be reversible. Thus, we tested the effects of the presence of thermostable RNase H (Fig. 4). The results showed that the presence of RNase H increased the effect of all of the tested oligonucleotides, especially that of Asp-2 (Fig. 4, ●). This suggests that Asp-2 temporarily bound to tRNA^{Asp} during the cooling process, and this hybrid was recognized and digested by RNase H. As tRNA^{Asp} retains its activity after the Asp-2 treatment without RNase H (Fig. 2, ●), the nearly complete inactivation with the aid by RNase H (Fig. 4, ●) suggests that almost all of the tRNA^{Asp} molecules were digested. Therefore, the inactivation in the presence of RNase H is irreversible. A treatment with *E. coli* RNase H after the annealing with Asp-3 was also tested and worked well (data not shown).

3.3. Digestion of the antisense DNA

Many antisense molecules have unpredictable, non-specific effects on cellular functions [18]. Thus, RQ1 RNase-free

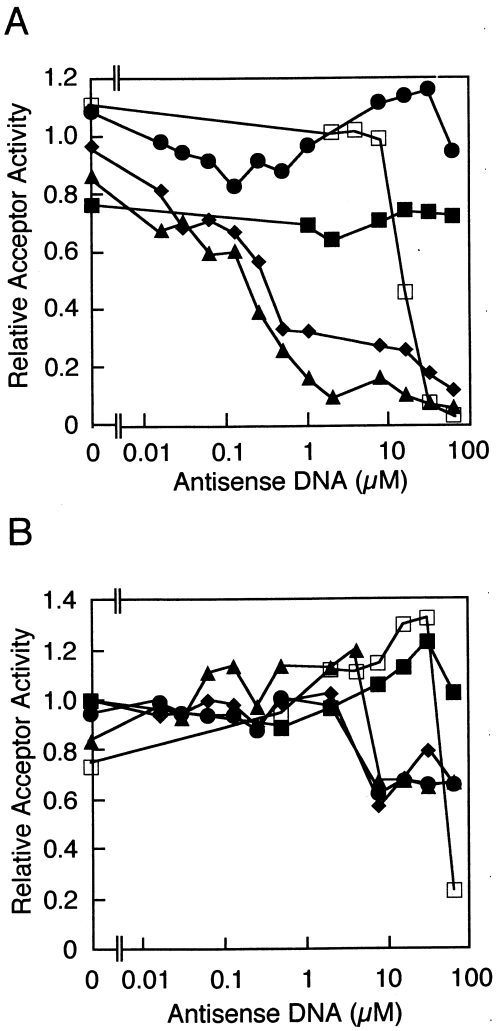

Fig. 2. Asp (A) and Phe (B) acceptor activities of the unfractionated *E. coli* tRNA after annealing with oligonucleotides complementary to $tRNA^{Asp}$. The amino acid acceptor activities after annealing, divided by those before annealing, are plotted against the oligonucleotide concentration. ■, Asp-1; ●, Asp-2; ▲, Asp-3; ◆, Asp-4; □, Asp-5.

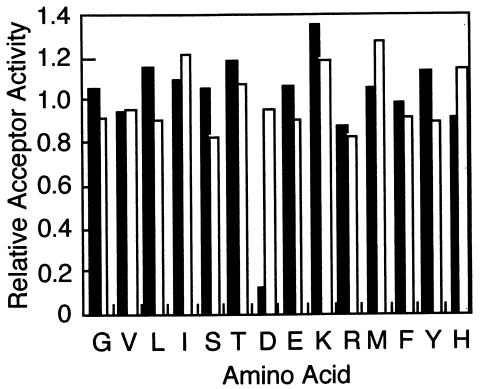

Fig. 3. Relative acceptor activities of the *E. coli* unfractionated tRNA for different amino acids after annealing with the Asp-3 oligonucleotide. Amino acid acceptor activities after annealing, divided by those before annealing, are shown. Filled bars, annealing with Asp-3; open bars, annealing without any oligonucleotide. The concentration of Asp-3 in the annealing mixture was 8 μM.

DNase I (Promega) (230 U/ml) was added to the tRNA mixture that had been treated with Asp-3 and RNase H. This mixture was incubated at 37°C for 1 h and was extracted with phenol/chloroform. The tRNA was precipitated with ethanol and dissolved in water. The acceptor activities of the 14 amino acids were not changed from those before DNase treatment (data not shown). We annealed this tRNA mixture again, without adding any antisense DNA, to allow $tRNA^{Asp}$ to refold and found that Asp acceptor activity was not restored (data not shown). Therefore, essentially all of the $tRNA^{Asp}$ molecules bound to the antisense DNA had been digested by the RNase H during the antisense treatment.

3.4. Cell-free translation of an mRNA lacking Asp codons using the $tRNA^{Asp}$-deficient mixture

This $tRNA^{Asp}$-deficient mixture (Fig. 5B) or the crude tRNA (Fig. 5A) was added to an S30-driven cell-free translation of an mRNA with an artificial coding sequence lacking Asp codons. Since the S30 contained a significant amount of tRNA, protein synthesis, as monitored by the incorporation of [^{14}C]Arg, was positive even when no tRNA was added (Fig. 5, ■). As the amount of the crude tRNA added to the reaction

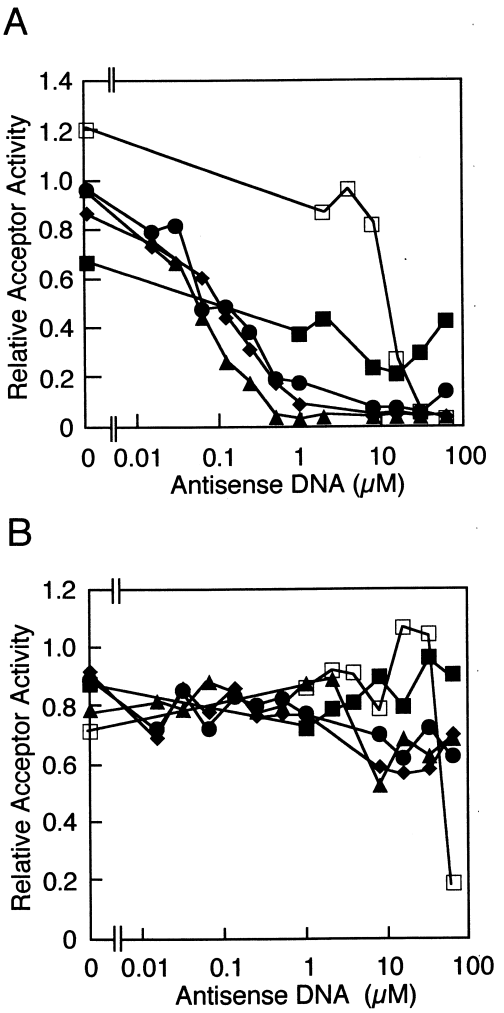

Fig. 4. Asp and Phe acceptor activities of *E. coli* unfractionated tRNA after annealing with $tRNA^{Asp}$-targeted oligonucleotides in the presence of *T. thermophilus* RNase H. The configuration of the figures is the same as in Fig. 2. ■, Asp-1; ●, Asp-2; ▲, Asp-3; ◆, Asp-4; □, Asp-5.

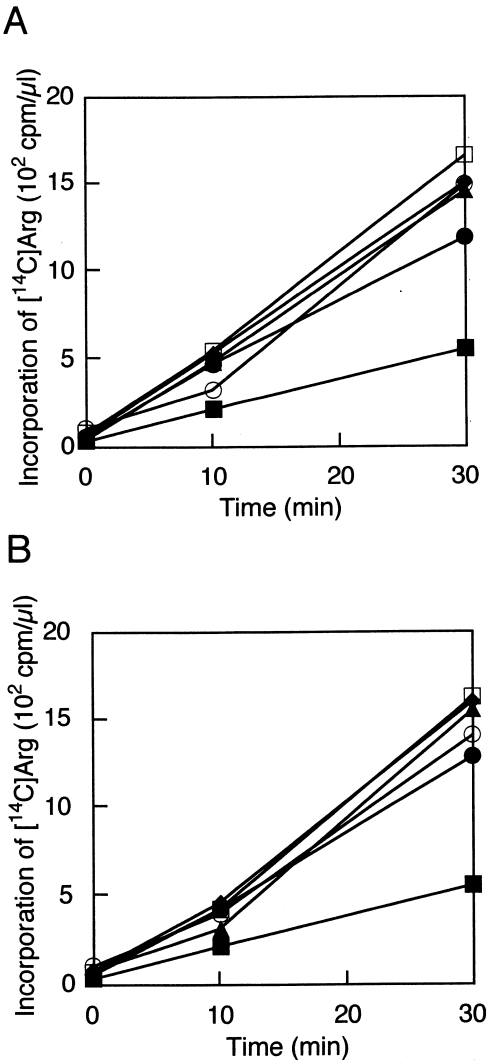

Fig. 5. Cell-free translation of an mRNA lacking Asp codons. *E. coli* crude tRNA (A) or Asp-3-treated tRNA (B) was added to a cell-free translation system, and the incorporation of the radioactive arginine into the protein fraction was measured. Concentrations of added tRNA in μM were: ■, 0; ●, 14; ▲, 27; ♦, 41; □, 54; ○, 68.

was increased, the protein synthesis slightly increased, reached a maximum at about 40 μM, and slightly decreased (Fig. 5A). This moderate dependence on the amount of added tRNA is generally observed for S30-based translation systems. A similar dependence, with a similar maximum at about 40 μM, was also observed when the $tRNA^{Asp}$-deficient mixture was added (Fig. 5B). Thus, this mixture functions normally, with regard to the translational efficiency, in this cell-free translation system.

3.5. Future aspects

The present results indicate that the oligonucleotide strategy is a promising method for the inactivation of a single tRNA species within an unfractionated *E. coli* tRNA mixture, although we had to test several candidates to find a useful oligomer. It is still unclear from our results whether we can find a specific and efficient oligomer and useful annealing conditions for any other tRNA species. This may be possible for most tRNA species, as a set of oligonucleotides can be used for specific detection of each tRNA species in *E. coli* by hybridization [11]. In addition, the specificity of the tRNA inactivation may be improved: lowering the concentration of an oligonucleotide while preserving the activity may be possible, in principle, by repeating the RNase H-aided hybridization/digestion of the target tRNA, with strict temperature control by a PCR apparatus. Knocking out multiple tRNA species may also be achieved by inactivating the individual tRNA species one by one.

The present 'knockout' tRNA can be the basis for preparing proteins with unnatural amino acid residues at multiple positions, and for integrating unnatural amino acids into polysome display peptide library systems [19]. These techniques may create new possibilities for protein engineering, peptide drug design, and combinatorial biochemistry. For these applications, an efficient translation system lacking gross tRNA still needs to be developed. The reconstitution methods developed for the kinetic analyses of protein synthesis [8] could be a prototype. These methods may also promote detailed analyses of the mechanism of protein biosynthesis and provide an experimental basis for research on the evolution of the genetic code.

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